Sexual dimorphisms in leukocyte trafficking in a murine peritonitis model.

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Summary Sentence: A discussion on the fundamental sex differences between male and female C57BL/6 mice and how this is reflected in inflammatory responses between the sexes.

Running title: Sex differences in leukocyte trafficking.

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Key Words: Inflammation, Sex Difference, Zymosan, C57BL/6, Monocyte, Neutrophil

Character Count:	**33979**
No. of Figures:	**7**
No. of Colour Figures:	**2**
No. of References:	**38**
No. of Words in Abstract:	**218**
No. of Words in Summary Sentence:	**24**

ABBREVIATIONS

ACK, ammonium-chloride-potassium

BM, bone marrow

C5aR, complement component 5a receptor

CCR, C-C chemokine receptor

CXCR, CXC chemokine receptor

CX_3CR1, CX3C chemokine receptor 1

F, female

G-CSF, granulocyte colony stimulating factor

IL-1(ra), interleukin 1 (receptor antagonist)

i.p., intraperitoneal

i.v., intravenous

M, male

M-CSF, macrophage colony stimulating factor

MFI, median fluorescence intensity

PRR, pattern recognition receptor

RFI, relative fluorescence intensity

sICAM-1, soluble intracellular adhesion molecule 1

TIMP-1, tissue inhibitor of metalloproteinases

TLR, Toll-like receptor

Tx, transfer

ABSTRACT

Sexual dimorphisms exist in the incidence and severity of many diseases, with females demonstrating relative protection from inflammatory conditions. The extent and mechanisms by which excessive leukocyte recruitment underlies these differences are not well established, and better understanding is essential for the development of targeted therapies. Here we set out to compare the male and female inflammatory response in a murine zymosan induced peritonitis model, and to understand how leukocyte subsets are mobilised from storage pools in both sexes. This work shows that female C57BL/6 mice recruit fewer classical monocytes and neutrophils during zymosan induced peritonitis. In addition, sex-differences were also evident in the circulation as female mice showed reduced neutrophilia and monocytosis versus male counterparts, despite having similar mobilisation from bone marrow (BM) stores. Importantly we show that storage and trafficking of splenic leukocytes during acute inflammation is distinct between the sexes. Male mice have greater splenic stores of neutrophils, classical and non-classical- monocytes, despite similar spleen sizes, signifying another source of potential pathogenic leukocytes. This work demonstrates that males and females have distinct leukocyte trafficking profiles in acute inflammation, and suggests that the spleen, not the BM, plays a role in determining sex-differences in the available pool of immune cells. Such dimorphisms demonstrate the importance of considering gender in assay development, drug design and clinical trials.

INTRODUCTION

Inflammation is the natural response to infection, tissue damage or irritation. In healthy individuals this complex process serves to clear harmful stimuli and initiate tissue repair. However, under certain circumstances this process can become dysregulated, causing an uncontrolled immune response that can lead to conditions such as sepsis. These conditions are often debilitating and impose considerable social and economic burdens on the global population. The inflammatory response involves both the endothelium and innate immune cells. The detrimental effects associated with inflammatory disease often stem from dysregulation of the mobilisation and subsequent recruitment of inflammatory innate immune cells from sites of storage to the infection. Although many of the drivers and mechanisms of inflammation are understood, the impact of sexual dimorphism on this process remains unclear.

Sex-differences in the inflammatory response have been described in diverse species. This is reflected in both the severity of the response but also the resultant tissue damage [1]. In human disease dimorphisms are regularly observed; men are more prone to inflammatory-type disorders such as ischemia-reperfusion injuries and sepsis, and autoimmune diseases such multiple sclerosis and rheumatoid arthritis are more prevalent in women. A possible explanation for these differences is the effect of hormones. Oestrogens for example are commonly proposed as protective in cardiovascular disease risk [2], although the exact mechanism is unclear.

There is also evidence that inherent differences in the immune system and innate cell phenotype are responsible for sexual dimorphisms in inflammatory responses. Female mice recruit fewer neutrophils upon detection of an inflammatory stimulus than aged-matched males. This corresponds with females having more resident leukocytes in the peritoneal and pleural cavities [3]. Additionally, female macrophages are more efficient at phagocytosis, ROS production and are more abundant in pathogen-sensing Toll-like receptors (TLRs) than male counterparts [3]. Reductions in these characteristics were achieved by ovariectomy signifying the influence of oestrogens on immune cell phenotype. These observations suggest females respond more effectively to, and clear infection faster than, males.

Although a mechanism for dampened female responses has been proposed, the reason for males having an exaggerated leukocyte recruitment response to an inflammatory stimulus is not clear. Such sex-differences have clinical implications in immune system disorders, for which male and female patients currently receive equal treatments. As targeting oestrogen signalling is an unlikely route for drug-development due to the obvious hormone-imbalance effect, this study sought to explore further male-female differences in the inflammatory response. We investigated sex-differences in the sources of recruited leukocytes by examining bone marrow and spleen storage pools, and probed further into the inflammatory response by also focusing on other leukocyte subsets using the well-characterised murine zymosan induced peritonitis model of resolving inflammation [4-6].

Under resting conditions, male mice had greater splenic reserves of neutrophils, classical and non-classical monocytes than aged-matched females. Previously observed sex-differences in neutrophil recruitment in peritonitis were confirmed in this study and extended to classical monocytes, which also accumulated to a greater extent in the peritoneal cavity of male mice. Despite mobilisation from bone marrow stores evident in response to the inflammatory stimulus, no sex-differences were observed. Males and females did however display different regulation of splenic neutrophil and monocyte trafficking in this model of inflammation. Uncovering such inherent differences in the male and female resident immune system and its response to infection should provoke more exploration into sex-differences in human responses. In doing so, this will move us a step closer towards the development of personalised sex-specific therapies which are clearly required.

MATERIALS AND METHODS

Experimental animals: All animal procedures were carried out in agreement with the United Kingdom Home Office Animals Scientific Procedures Act 1986 under project licence 70/7884. C57BL/6 mice, 8-11 weeks old, were used in this study (22-30g males, 18-25g females; Charles River Laboratories) and were housed in individually ventilated cages with a controlled 12 hour light/dark cycle (lights on 0700 -1900), and constant temperature (21±2°C). Mice from the same litter were equally distributed between experimental groups and males and females were treated at the same time. In some experiments mice heterozygous for targeted replacement of the CX_3CR1 gene with cDNA encoding eGFP ($CX_3CR1^{+/GFP}$) were used [7].

Induction of peritonitis and collection of leukocytes: Peritonitis was induced by intraperitoneal (i.p) injection of 1mg zymosan A (lot #BCBF4506V, Sigma) in 0.5ml sterile PBS after vortexing and 15s sonication as previously described [4]. After test periods the animals were anaesthetised by an intramuscular injection of 1ml/kg ketamine (40mg)/ xylazine (2mg) in saline, and were sacrificed by cervical dislocation with care not to tear the carotid vessels. Leukocytes were obtained from blood by cardiac puncture using a 27G needle and syringe containing 50µl 0.5M EDTA; from the peritoneal cavity, by lavage with 6ml wash buffer (PBS + 0.25% BSA + 2mM EDTA); from bone marrow, by flushing femurs through with ice-cold FACS buffer (PBS + 1% goat's serum), and from the spleen, by grating on a 40µm cell strainer followed by washing through with cold FACS buffer. Blood and spleen samples were subject to erythrocyte lysis with ACK lysis buffer (150mM NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA in dH_2O, pH7.3), and all samples were washed twice with ice-cold FACS buffer by centrifugation (5 min, 300g, 4°C).

Flow cytometry: Total leukocytes were counted using a haemocytometer with trypan blue (Sigma) for exclusion of dead cells, and adjusted to a concentration of $1x10^6$/ml in FACS buffer. $1x10^5$ cells were mixed with 0.5µg anti-mouse CD16/CD32 Fc-block (BD Pharmingen) for 20 min on ice. Cells were immuno-stained for 30 min on ice in the dark with fluorescently conjugated antibodies to different leukocyte subsets including CD3 (clone 17A2), B220 (clone RA3-6B2), F4/80 (clone BM8) (all Biolegend), CD115 (clone AFS98), and Gr1 (clone RB6-8C5). Antibodies for the

detection of murine cell surface receptors were included in certain experiments: TLR2 (clone T2.5), TLR6 (clone 4186-1), CCR1 (clone 643854), CCR2 (475301) (all R&D Systems), dectin-1 (clone bg1fpj), CXCR4 (clone 2B11) (eBioscience), CXCR2 (clone TG11), CCR3 (clone J073E5), CCR5 (clone HM-CCR5), C5aR (clone 20/70) (all BioLegend) or respective isotype antibodies as controls. Cells were then washed twice by centrifugation (5 mins, 300g, 4°C) and resuspended in 200µl of FACS buffer for analysis by flow cytometry. Samples were acquired on the LSRFortessa™ (BD Biosciences) using FACSDiva™ software (BD Biosciences), and compensation set using single stained cells or anti-rat Ig, κ CompBeads (BD Biosciences). Data was analysed using FlowJo (v7.6.4).

Chemokine and cytokine arrays: Chemokine and cytokine levels in peritoneal lavage from male and female mice in response to i.p. zymosan were measured using Proteome Profiler™ Mouse Cytokine kit (panel A) and Mouse Chemokine Antibody Arrays according to the manufacturers' instructions (R&D Systems). The resultant blots were scanned and quantified by densitometry using IMAGEJ.

Leukocyte transfer (Tx): Leukocytes were isolated from the bone marrow of aged-matched untreated donor male and female C57BL/6 mice and were treated with ACK lysis buffer (5 min, room temperature). Male and female cells were labelled ex vivo with CellTracker™ Orange and CellTracker™ Violet dyes (2µM, Life Technologies), respectively. $10x10^6$ labelled donor leukocytes ($5x10^6$ male + $5x10^6$ female) were transferred (i.v.) into recipient male or female mice. After 4 hours recipient mice were culled by cervical dislocation and spleen leukocytes harvested. Leukocytes were counted on a haemocytometer, labelled with antibodies recognising CD115 and Gr1 and samples were analysed by flow cytometry.

Statistical analysis: Data was expressed as mean ± SEM. Statistical analysis was carried out using Prism 4.0 (GraphPad). For comparisons between multiple groups a 1-way ANOVA was performed, followed by the Bonferroni post-test for multiple comparisons. For comparisons between 2 groups, the 2-tailed unpaired Student's *t*-test was used.

RESULTS

Sex differences in leukocyte subset numbers under naïve conditions: A number of studies have reported differences in inflammatory responses between males and females [3, 8-10]. These studies have predominantly looked at cytokine levels and neutrophil responses to various stimuli. Here we set out to expand these observations by comparing other leukocyte subsets between aged-matched male and female mice. In an initial experiment we established that there is no significant difference in total circulating, bone marrow, or spleen leukocyte counts between male and female C57BL/6 mice (**Figure 1A**). In line with previous reports [3], females exhibited significantly more peritoneal leukocytes than aged-matched males (**Figure 1A**, 4.4±0.20 $x10^6$ vs 3.3±0.20 $x10^6$). This was found to be the result of significantly more peritoneal macrophages in females as compared to males (**Figure 1B**). In order to differentiate the populations of leukocyte subsets we used C57BL/6 mice heterozygous for eGFP under the control of the CX_3CR1 promoter. Inflammatory responses in these reporter mice are identical to wild type C57BL/6 mice [7]. Monocyte subsets could therefore be detected by flow cytometry in conjunction with the anti-Ly6C/Ly6G antibody Gr1 in leukocyte preparations from blood, the peritoneal cavity, bone marrow and spleen. In alignment with previous studies [11] classical monocytes were identified as $CX_3CR1^+Gr1^+$, non-classical monocytes as $CX_3CR1^+Gr1^-$ and neutrophils were $Gr1^{high}$. In addition, an indication of B and T cell numbers was achieved using B220 (CD45R) and CD3 as markers respectively (gating strategies are defined in **Supplemental Figure 1**). Using this approach we were able to compare numbers of leukocyte subsets from different compartments in male and female mice (**Figure 1**). For the most part neutrophils, classical and non-classical monocytes, $B220^+$ cells and $CD3^+$ cells showed no significant differences in the blood, peritoneal cavity and bone marrow (**Figure 1 C-G**). Female peritoneal $B220^+$ cells were however more numerous than males, in line with other studies [3], and contributed to the greater numbers of total leukocytes found in the female peritoneum (**Figure 1F**). Interestingly significantly fewer neutrophils, classical and non-classical monocytes were present in the female spleen as compared to the male (**Figure 1C and D**, neutrophils: 18.7±2.00 $x10^5$ vs 44±7.94 $x10^5$; classical monocytes: 7.7±0.82 $x10^5$ vs 16.1±2.49 $x10^5$; non-classical monocytes: 6.6±0.75 $x10^5$ vs 14.5±1.75 $x10^5$). These differences were still evident

even when spleen sizes and body weights were taken into account (**Table 1**). Together these data suggest that there are sex differences with regard to basal leukocyte subset numbers in C57BL/6 mice.

Reduced monocyte and neutrophil responses in females during zymosan induced peritonitis: Zymosan induced peritonitis is an established model of inflammation [4]. Lower doses (1mg) elicit a transient inflammation whereas higher doses (10mg) result in an aggressive response. To build on our previous observation of sex differences in leukocyte subsets under naïve conditions, we compared the response of leukocytes in a mild resolving inflammatory scenario induced using a low 1mg dose of zymosan. Analysis of leukocyte recruitment to the peritoneal cavity revealed that significantly fewer neutrophils were recruited to the site of inflammation in females as compared to males 3 hours after zymosan injection (**Figure 2A**; neutrophils: 8.0±0.91 $x10^6$ vs 11.4±1.12 $x10^6$). Subsequent measurement of neutrophils over a full time course revealed that despite females demonstrating a dampened response, there was a clearance of neutrophils by 96h in both sexes (**Figure 2A**). Clearance of neutrophils at this time point is concurrent with previous reports [4] and indicative of resolution of inflammation. More classical monocytes were recruited after 3 hours in male mice (2.7±0.35 $x10^5$ vs 1.52±0.21 $x10^5$) but this was not so of non-classical monocytes which declined in number roughly 2.5 fold in both sexes (**Figure 2B and C**). Other leukocyte subsets including $B220^+$ and $CD3^+$ cells were not significantly different at this time point (**Figure 2D and E**). Although there were a greater number of peritoneal macrophages in naïve females, the macrophage levels in both sexes was substantially depleted 3 hours after injection of zymosan (**Figure 2F**).

Receptor expression and chemokine microenvironment: Since we observed sex differences in neutrophil and classical monocyte recruitment to the peritoneal cavity in response to zymosan peritonitis, we next tested whether there were differences in the ability of male or female peritoneal macrophages to sense and respond to the inflammatory stimuli as has previously been proposed [3]. Detection of i.p. zymosan occurs through the pattern recognition receptors (PRRs) TLR2, TLR6 and dectin-1. The TLR2-TLR6 heterodimer is required for the inflammatory response and activation of NFκB, and dectin-1 has roles in zymosan phagocytosis [12, 13]. No major differences were found in the expression of these receptors on the surface of

male and female peritoneal macrophages (**Figure 2G**), suggesting there are no sex differences in the ability of males and females to sense i.p. zymosan.

Immune cells release a broad spectrum of chemokines and cytokines in response to inflammatory stimuli. We next examined whether the sex differences seen in neutrophil and classical monocyte recruitment could be explained by variances in the release of chemokines and cytokines in the tissue in response to zymosan. We used Proteome Profiler^{TM} arrays to measure the levels of a broad spectrum of chemokines and cytokines in peritoneal lavage from naïve (**Supplemental Figure 2**) and inflamed mice. Peritoneal injection of zymosan resulted in a substantial increase in inflammatory mediators such as C5a, G-CSF, sICAM-1, IL-1β, IL-1ra, IL-6, CXCL10, CXCL1, M-CSF, CCL2, CCL4, CXCL2 and TIMP-1. Other mediators such as CCL6, chemerin, IL-16, CXCL1, CCL8, CCL12 and CCL9/CCL10 were also released in response to zymosan. There appeared to be no appreciable differences in the cytokine and chemokine profiles released in response to zymosan between males and females (**Figure 2H and I, Supplemental Figure 2**).

Males recruit more neutrophils and classical monocytes to the blood during zymosan peritonitis despite no sex differences in leukocyte bone marrow mobilisation: Since we could not observe any major differences in the peritoneal microenvironment between male and female mice, we next asked whether the differences observed in peritoneal leukocyte recruitment was related to the mobilisation of leukocytes to the blood. Levels of circulating neutrophils and classical monocytes increased greatly 3 hours after zymosan administration and returned to naïve levels after approximately 24 hours (**Figure 3A and B**). Interestingly, and as seen with recruitment to the peritoneal cavity, circulating neutrophils and classical monocytes were significantly fewer in females as compared to males 3 hours after the administration of zymosan (**Figure 3A and B**). The profile of circulating non-classical monocytes over time suggests there was an increase in male circulating numbers after 3 hours, however this was not observed in females (**Figure 3C**).

The bone marrow is a major leukocyte store in both mice and humans and is reportedly utilised in peritonitis [14]. We speculated that the differences observed in neutrophil and classical monocyte numbers in both the blood and peritoneal cavity in response to zymosan could be due to dimorphisms in the way leukocytes are

mobilised from the bone marrow. CXCR4 plays as key role in this process [15] and we first established that there were no differences in the expression of this molecule between male and female bone marrow leukocytes (**data not shown**). We then counted the number of leukocytes remaining in the bone marrow (from the right femur) after i.p. zymosan. After 3 hours the number of bone marrow neutrophils and classical monocytes declined substantially, with levels restored to that of the naïve state roughly 72 hours post injection (**Figure 3D and E**). This indicated a rapid mobilisation of both cell types in response to zymosan which was to the same extent in males and females. Mobilisation of non-classical monocytes from the bone marrow was equivalent in males and females, however was not as substantial as the classical subset (**Figure 3F**). Levels of non-classical monocytes were replenished to naïve levels after 72 hours. These data indicate that there are more circulating monocytes and neutrophils in males but no sex differences in mobilisation of these cells from the bone marrow during early zymosan peritonitis.

Sex differences in monocyte and neutrophil mobilisation from the spleen: The spleen is another large store of leukocytes and has been shown as a major source of classical monocytes in certain inflammatory conditions [16-19]. We next set out determine whether there were any sex differences in the way monocytes and neutrophils are trafficked to and from this organ. We measured leukocyte numbers in the male and female spleen in both naïve and zymosan-treated mice (i.p., 3h). Significantly fewer neutrophils, classical and non-classical monocytes were present in the spleen of naïve female mice as compared to males, as already observed (**Figure 4A-C**). Zymosan had no effect on splenic neutrophil numbers however the number of female neutrophils roughly doubled in the spleen at this time point (**Figure 4A**). Male classical monocyte numbers were greatly reduced 3 hours post zymosan injection, however female monocytes remained roughly consistent (**Figure 4B and C**). These data suggest that males may utilise a splenic store of monocytes during inflammation and this may also account for the higher numbers of monocytes being recruited to the peritoneal cavity. Of importance, B and T cells represent the most abundant cell type in the spleen, and similar numbers are present in naïve and zymosan-treated (i.p., 3h) male and female mice (**Figure 4D and E**). This suggests that the observed sex differences relate primarily to neutrophils and monocytes.

Sex differences in basal splenic leukocyte numbers is not related to the splenic chemokine microenvironment or chemokine receptor expression: To understand the underlying reason for more neutrophils and monocytes in the male compared to the female spleen, we first compared the basal splenic cytokine micro-environments. Using Proteome Profiler™ cytokine arrays we showed that although a number of chemokines and inflammatory mediators were in abundance e.g. CCL5, CXCL9, sICAM-1, IL-1ra, CXCL10 and IL-1α, there were no sex differences (**Figure 5A**). In addition, it revealed the presence of those molecules with neutrophil or monocyte chemotactic capabilities such as CCL2, CCL3, CCL5, C5a, CXCL1 and CXCL2, which were present in vastly ranging relative quantities, but to similar extents in both sexes. The expression of the corresponding receptors to these inflammatory mediators on leukocyte subsets from the spleens of male and female mice were therefore assessed to offer explanation for the observed sex differences in splenic leukocyte composition.

CXCL1 and CXCL2 are potent neutrophil-attracting chemokines that exert their functions via CXCR2 [20]. Correspondingly, CXCR2 was highly expressed on the splenic neutrophil cell surface, and expressed less on the surface of the other leukocyte subsets (**Figure 5B-D**). No significant difference between the expression of CXCR2 on the surface of male and female splenic leukocytes was observed ($p>0.05$). CXCL12 on the other hand acts on CXCR4 on neutrophils, but also on monocytes, to retain these cells in the bone marrow. CXCR4 was expressed at similar levels on the surface of neutrophils, monocyte subsets, and B cells from the spleen (**Figure 5B-D**). Non-classical monocytes from the female spleen however, had significantly ($p<0.05$) higher levels of CXCR4 than those from the male spleen (female MFI: 3003±359.6; male MFI: 1821±108.9, **Figure 5D**).

Chemokines CCL2, CCL3, and CCL5 were all detectable, albeit at low levels, in the mouse spleen as determined by cytokine array. CC chemokines are more promiscuous than those containing the CXC motif and exert their effects by binding to a selection of CC chemokine receptors [21]. CCR1, CCR2, CCR3 and CCR5 represent key monocyte chemokine receptors that are associated with the above ligands [22]. CCR1 represents a receptor for an array of ligands primarily chemotactic for monocytes, but in some cases for neutrophils also [23]. Consequently, CCR1 was most highly expressed on the monocyte subsets and

neutrophils. CCR2 is the major receptor responsible for chemotaxis of classical monocytes toward CCL2 [24, 25]. In the spleen, CCR2 was most highly expressed on classical monocytes, followed by the non-classical subset, with very low expression on neutrophils (**Figure 5B-D**). CCR3 is the receptor for chemokines including CCL5, CCL7, CCL11 and CCL13, and CCR5, the receptor for chemokines including CCL3, CCL4 and CCL5, showed low expression on all splenic leukocyte subsets studied (**Figure 5B-D**). Notably, no sex-differences in the expression of CCR1, CCR2, CCR3, or CCR5 were evident on splenic leukocyte subsets with the exception of non-classical monocyte CCR2 which was expressed more on male (MFI: 20.6±1.01 $x10^3$) versus female cells (MFI: 13.0±1.69 $x10^3$) (p<0.01) (**Figure 5D**).

Finally we compared the expression of complement component C5a receptor (C5aR), between splenic leukocytes in males and females. C5a is a highly inflammatory peptide and represents an effective chemoattractant for neutrophils and monocytes via C5aR [26, 27]. C5aR was highly expressed on splenic neutrophils with moderate monocytic expression and with no apparent sex difference (**Figure 5B-D**). Sex-differences were evident on non-classical monocytes with female cells exhibiting significantly (p<0.01) higher expression of C5aR versus male cells (MFI: 4.6±0.35 $x10^3$ vs 2.8±0.34 $x10^3$, **Figure 5C**).

Inherent properties of male and female neutrophils and classical monocytes are likely to underlie sex differences in splenic leukocyte composition. To understand whether properties intrinsic to male and female leukocytes, or properties relating to the male and female body, account for increased leukocyte numbers in the male spleen, leukocyte transfer experiments were performed.

Equal numbers of male and female donor leukocytes were transferred to male and female recipient mice (i.v.) and their accumulation in the spleen was assessed. We proposed that equal accumulation of donor male and female neutrophils and monocytes in the recipient spleens would suggest that inherent properties of the spleen and body (eg. chemokines and hormones) underlie the observed sexual dimorphisms in splenic leukocyte composition. Conversely, different accumulation of male compared to female donor leukocytes in the recipient spleen would be suggestive of inherent differences in the phenotype of the male and female

neutrophil and monocyte. Comparable numbers of male (M) and female (F) total donor leukocytes accumulated in male recipient (M→M: 50±10.3 $x10^4$; F→M: 66±31.2 $x10^4$) and female recipient (M→F: 67±11.8 $x10^4$; F→F: 73±20.5 $x10^4$) mice (**Figure 6A**), suggesting no adverse reactions in cross-sex donation. Irrespective of the sex of the recipient, greater numbers of donor male neutrophils accumulated in the recipient spleen (M→M: 15±5.8 $x10^4$; M→F: 19±4.3 $x10^4$), compared to female donor cells (F→M: 9±3.5 $x10^4$; F→F: 12±3.2 $x10^4$, **Figure 6B**). This was also the case with classical monocytes (M→M: 5±0.7 $x10^4$; M→F: 7±1.3 $x10^4$; F→M: 3±0.6 $x10^4$; F→F: 4±0.9 $x10^4$, **Figure 6C**). The extent of donor neutrophil or classical monocyte accumulation was similar with male and female recipients (**Figure 6B and C**).

These data suggest that specific, and currently unknown, properties of male neutrophils and classical monocytes make them more amenable to homing to the spleen than equivalent female cells.

DISCUSSION

Substantial differences exist in the incidence and severity of certain inflammatory disorders with males more prone to conditions with excessive or uncontrolled leukocyte recruitment and activation underlying their pathology (i.e. sepsis, ischemia/reperfusion injuries). The mechanisms behind these dimorphisms are unclear, however previous studies report and link fewer resident macrophages expressing fewer PRRs to greater neutrophil recruitment in male mice in the zymosan peritonitis model [3]. In this study we expand upon this current knowledge of sexual dimorphisms in immune cell trafficking in acute inflammation. We show that like neutrophils, classical monocytes also accumulate to a greater extent in male C57BL/6 mice during zymosan peritonitis. Previous studies have shown that infiltration of classical monocytes is independent of neutrophil migration in peritonitis [28], suggesting our observed sex differences in classical monocytes are not a result of the dimorphic neutrophil phenotype.

We hypothesised that like previous studies, the dimorphisms seen were due to differences in immune cell phenotype and number. Whilst female mice did exhibit more peritoneal macrophages, we found no sex difference in the zymosan-sensing apparatus (TLR2, TLR6, dectin-1), contradictory to other studies showing female macrophages to be higher in TLR2 [3]. Cytokines and chemokines also play a pivotal role in the immune response via the governing of leukocyte trafficking. As sex differences in cytokine production have previously been reported [29, 30], we examined the peritoneal microenvironment for differences in the ability of male and female mice to generate inflammatory mediators. Peritoneal cytokine microenvironments after zymosan were found to be comparable in male and female mice suggesting equivalent chemotactic signal generation, and providing no explanation for sex-differences in recruitment. We show the root of these sex differences in zymosan peritonitis is in fact upstream of the transmigration process as males also had more circulating neutrophils and classical monocytes. Of note, the presence of these sex differences in both the blood and tissue is also suggestive of similar transendothelial migration mechanisms and kinetics between the sexes. Collectively, a difference in the phenotype and function of resident peritoneal cells did not underlie the sex-differences observed.

In response to inflammatory stimuli, leukocytes are mobilised from cellular storage pools, e.g. the bone marrow, to participate in the inflammatory response [15, 31]. In line with previous studies [14], the bone marrow indeed mobilised neutrophils in response to zymosan. We added to this mechanism by demonstrating the same is true for monocytes. This storage pool was the major leukocyte contributor but released neutrophils and monocytes to equal extents in male and female mice. The spleen also represents a large site of leukocyte storage [32]. Importantly, we identify this organ as a dimorphic pool of leukocytes with respect to both storage and trafficking. Male mice were found to have greater basal stores of neutrophils and monocytes in their spleen compared to female counterparts. Interestingly, spleen sizes were comparable in males and female mice indicating males do not have more splenic stores due to differences in organ size.

In response to zymosan, neutrophil and classical monocyte trafficking in respect to the spleen is inherently different, with mobilisation of splenic classical monocytes in male mice and accumulation of neutrophils in the female spleen (**Figure 7**). In line with the observed classical monocyte mobilisation in males, the spleen has previously been identified as a source of monocytes in a variety of inflammatory conditions including atherosclerosis [17], myocardial infarction [16], and spinal cord injury [19], although the results in these studies did not discriminate by sex. The function of neutrophil trafficking in females is however unclear. Involvement of cross-talk with the adaptive immune system may provide an explanation. Whether these cells in any way contribute to the previously described splenic B-helper neutrophils, which enhance innate immunoglobulin responses to microbial products, would need to be investigated [33]. We hypothesise the traffic of neutrophils and classical monocytes to and from the spleen during zymosan peritonitis accounts for the overall sex differences observed in leukocyte recruitment, with splenic classical monocytes contributing to the additional recruited cells in male mice. Therefore, whilst the spleen has been previously implicated as a source of classical monocytes in certain inflammatory conditions, this is the first description of differential deployment of these cells in males and females.

In healthy individuals monocytes and neutrophils are produced in the bone marrow, yet the mechanisms resulting in the presence of mature classical monocytes in the spleen are unclear. We hypothesised a more chemotactic spleen microenviroment,

or higher chemokine receptor expression, may underlie the greater numbers of neutrophils and monocytes in the male spleen. This was not the case as no major sex differences in the splenic cytokine/chemokine environment under homeostatic conditions were found. The expression of major neutrophil- and monocyte-associated chemokine receptors (CCR1, CCR2, CCR3, CCR5, CXCR2, CXCR4, C5a) were expressed at equivalent levels on male versus female splenic neutrophils and classical monocytes. Against this trend was male non-classical monocytes which had greater expression of CCR2, but lower expression of complement component C5a receptor 1 (C5aR) and CXCR4. Higher CCR2 on male cells could provide explanation for more of these cells in the male versus female spleen, but leaves questions for the origins of the classical subset which showed no differences in this receptor. The exact reasons for sex-differences in non-classical monocytes are unclear, and may relate to the development of these cells. It is highly debated whether non-classical monocytes are an independent monocyte subpopulation, or whether they represent a classical monocyte precursor. Furthermore, whilst it is clear a population of undifferentiated bone fide monocytes reside in the spleen, it is not clear whether other populations exist to replenish the various splenic macrophage populations, or whether these cells are derived from embryonic precursors and self-renew [16, 34, 35].

The mechanisms underlying the observed sex-differences in splenic neutrophil and monocyte numbers therefore remains unclear. Consequently, we reasoned that fundamental localisation of such cells in specific organs under basal conditions is unlikely to be explained by a single molecule or receptor. Leukocyte transfer experiments revealed that neutrophils and classical monocytes from male donors homed to the spleen of recipient mice in greater numbers than those from female donors. This was irrespective of the sex of the recipient and receiving spleen. The enhanced ability of male neutrophils and classical monocytes to home to recipient mouse spleens suggests it is inherent characteristics of these cells, not the properties or environment of the spleen, which underlies the sex-differences in basal splenic pools of these cells. A more detailed comparison, e.g. by the use of proteomics or gene array, of male and female neutrophils and classical monocytes would need to be carried out to identify the characteristics underlying these dimorphisms. Whilst the leukocyte transfer experiments identify clear trends, these

data are on the whole not significant. This suggests there are indeed contributing factors from the 'body', e.g. hormones, that are also influencing phenotype, function, and trafficking of neutrophils and classical monocytes. 17β-oestradiol is one such candidate which has been reported to have 'immune-damping' effects, particularly with respect to PRRs, adhesion molecules and cytokine production [29, 36-38]. Interestingly, non-classical monocytes were detected only in negligible numbers in the spleens of recipient mice. This could be due to different chemokine expression profiles of these monocytes, possibly as a result of a different maturity state compared to the classical subset, meaning they are less amenable to splenic homing. Overall the differences in the naïve mouse spleen are likely due primarily to inherent differences between the male and female leukocytes.

Overall, this study has shown that sex-differences in the inflammatory response are not restricted neutrophil recruitment, extending to classical monocytes also. We have shown the spleen represents a sexually dimorphic pool of leukocytes in C57BL/6 mice, and differences in splenic neutrophil and monocyte trafficking likely underlie differences in leukocyte recruitment in zymosan peritonitis. Since C57BL/6 mice are used widely in characterising inflammatory responses the observations reported here are of paramount importance both for the design and interpretation of experiments. Whether, this is the case for other animal models, and indeed for humans, still needs to be determined. Should this work translate to the human, the spleen leukocyte pool may well represent a therapeutic target for diseases with aberrant leukocyte recruitment as the underlying pathogenesis.

AUTHORSHIP CONTRIBUTIONS

E.K., R.S and J.R.W conceived of the work and designed the experiments. E.K. and L.G. performed the experimental work and in conjunction with J.R.W. drafted the manuscript.

ACKNOWLEDGEMENTS

E.K was funded by the British Heart Foundation Grant # FS113028789 and JRW was funded by Arthritis Research-UK Grant #19207.

CONFLICT OF INTEREST DISCLOSURE

The authors can confirm there is no conflict of interests arising from this work.

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FIGURE LEGENDS

Figure 1; Sex-differences in murine basal leukocyte subset numbers from different compartments. Numbers of cells corresponding to the indicated leukocyte subsets were counted from male (■) and female (□) C57BL/6 mice. Comparisons were made from leukocytes prepared from the blood, the peritoneal cavity, bone marrow (BM) and spleen. Total leukocytes are shown in (A), peritoneal macrophages ($F4/80^+$) in (B), neutrophils ($Gr1^{high}$) in (C), classical monocytes ($CX_3CR1^+Gr1^+$) in (D), non-classical monocytes ($CX_3CR1^+Gr1^-$) in (E), $B220^+$ B cells in (F) and $CD3^+$ T cells in (G). n numbers for each measurement are shown below the bars in each graph. Data is shown as mean ± SEM and significant differences between sexes were determined by using Student's *t*-test and indicated by * (p<0.05), ** (p<0.01), *** (p<0.001).

Figure 2; Sex differences in leukocyte recruitment to the peritoneal cavity in response to zymosan induced peritonitis. Male (■) and female (□) C57BL/6 mice were treated with or without i.p. zymosan. (A) Numbers of neutrophils ($Gr1^{high}$ cells) were measured over a period of 96 hours. Data are shown as mean ± SEM of at least 14 (0h), 16 (3h), 10 (24h) and 3 (48h, 72h, 96h) mice. Sex differences in the accumulation of neutrophils in the peritoneal cavity at the indicated time points were determined by Student's *t*-test. Significance in cell numbers in male compared to female mice is indicated by * (p<0.05). (B) Numbers of male and female classical monocytes ($CX_3CR1^+Gr1^+$), (C) non-classical monocytes ($CX_3CR1^+Gr1^-$), (D) $B220^+$ cells, (E) $CD3^+$ cells, and (F) macrophages ($F4/80^+$ cells) were also determined after 3h of peritonitis. Comparison of recruitment in male and female mice was evaluated using Student's *t*-test, with significant differences at 3h indicated by * (p<0.05) or ** (p<0.01). Data is displayed as the mean ± SEM and n numbers for each measurement are shown below the bars. (G) Expression of the zymosan receptors; TLR2, TLR6 and Dectin-1 in peritoneal macrophages from male (■) and female (□) C57BL/6 mice. Expression of TLR2 (n>18), TLR6 (n>14) and dectin-1 (n>11) was evaluated as relative fluorescence intensity (RFI) to an isotype antibody. Data is shown as mean ± SEM and significant differences between sexes was determined by a Student's *t*-test * (p<0.05). (H) Peritoneal cytokine profile in inflamed and naïve peritoneum. Representative Proteome Profiler™ array blots of peritoneal lavage from

naïve and zymosan injected (1mg, 3h) male and female C57BL/6 mice. (I) Semi-quantification of Proteome Profiler™ blots by densitometry, data shown is the mean of three experiments and error bars reflect the S.D.

Figure 3; Sex differences in circulating leukocytes in response to zymosan peritonitis. Zymosan was administered to male (●) and female (○) mice to induce peritonitis. Numbers of (A) neutrophils ($Gr1^{high}$ cells), (B) classical monocytes ($CX_3CR1^+Gr1^+$) and (C) non-classical monocytes ($CX_3CR1^+Gr1^-$) in the blood were counted at 0h (untreated), 3h, 24h, 48h, 72h, and 96h. Data is presented as mean ± SEM of at least 14 (0h), 16 (3h), 10 (24h) and 3 (48-96h) mice for each sex. Numbers of (D) neutrophils, (E) classical monocytes and (F) non-classical monocytes were counted from the right femur bone marrow at the indicated time points. Data is presented as mean ± SEM of 8 (0h), 13 (3h), 9 (24h) and 3 (48-96h) mice of each sex. Sex-differences at each time point were determined by Student's *t*-test and significance indicated by * (p<0.05), ** (p<0.01), and *** (p<0.001).

Figure 4; Splenic leukocyte trafficking during inflammation. Splenic (A) neutrophils ($Gr1^{high}$ cells), (B) classical monocytes ($CX_3CR1^+Gr1^+$), (C) non-classical monocytes ($CX_3CR1^+Gr1^-$), (D) $B220^+$ and (E) $CD3^+$ cells numbers were determined in male (■) and female (□) mice treated with or without zymosan i.p. for 3h. The influence of zymosan treatment on splenic leukocyte subset numbers was determined by Student's *t*-test and significance indicated by * (p<0.05) and ** (p<0.01). Data are expressed as mean ± SEM and n numbers are indicated below bars.

Figure 5; Basal splenic chemokine micro-environment and leukocyte receptor expression. (A) The chemokine profile of splenic lysates from untreated male (■) and female (□) C57BL/6 mice were compared using a Proteome Profiler™ chemokine array. Blots were quantified by densitometry and the data shown is the mean of experiments with error bars reflecting the highest and lowest values obtained. (B-D) Leukocytes were isolated from male and female spleens and cell surface expression of the CC chemokine receptors CCR1, CCR2, CCR3, CCR5, complement component C5a receptor (C5aR) and the CXCR2 and CXCR4 chemokine receptors were measured on (B) neutrophils, (C) classical monocytes

and (D) non-classical monocytes. Receptor expression is displayed as median fluorescence intensity (MFI) as data is the mean ± SEM (n=6). Sex differences in receptor expression was determined by Student's *t*-test and significance indicated by *($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

Figure 6; Trafficking of donor transfer leukocytes to recipient spleens. (A) Leukocytes were isolated from the BM of donor male and female C57BL/6 mice and cells were labelled ex vivo with CellTracker™ Orange and CellTracker™ Violet dyes (2μM, Life Technologies), respectively. $10x10^6$ labelled donor leukocytes ($5x10^6$ male + $5x10^6$ female) were transferred (i.v.) into recipient male or female mice. After 4h recipient mice were culled by Schedule 1 (cervical dislocation) and spleen leukocytes harvested. Leukocytes were counted on a haemocytometer and labelled with fluorescently conjugated antibodies to CD115 and Gr1. Samples were analysed by flow cytometry. (B) Donor leukocytes in the recipient spleen. Male donor cells were identified as CellTracker™ Orange$^+$ and female donor cells as CellTracker™ Violet$^+$. (C) Donor neutrophils in the spleen were identified by subsequent gating on Gr1^{high} cells. (D) Donor classical monocytes in the spleen were identified by subsequent gating on CD115$^+$Gr1$^+$ cells. Data are mean ± SEM, n=7/6/6/5. Differences in the accumulation of donor leukocyte subsets in recipient spleens were determined by 1-way ANOVA with Bonferroni's post-test for multiple comparisons.

Figure 7; Sex differences in leukocyte trafficking in C57BL/6 mice in response to zymosan induced peritonitis. (A) Under naïve conditions there are equal numbers of leukocyte subsets between male and female C57BL/6 mice in the bone marrow and the circulation but not in the spleen, where males have higher numbers of neutrophils and classical monocytes than females. (B) During zymosan induced peritonitis more neutrophils and classical monocytes are recruited to the peritoneal cavity in males (1). In addition circulating neutrophils and classical monocytes are more abundant in males (2), however mobilisation of these cells from the bone marrow is the same between males and females (3). Interestingly more classical monocyte mobilisation from the spleen occurs in males whereas greater numbers of neutrophils are recruited to the spleen in females (5).

Table 1

	Male	Female	*p* value
Weight (g)	25.0 ± 2.58 (n=99)	20.2 ± 2.16 (n=94)	<0.001
Femur (mg)	81.6 ± 17.00 (n=9)	76.4 ± 27.18 (n=6)	NS
Femur: body weight (mg/g)	3.2 ± 0.74 (n=9)	3.80 ± 1.42 (n=6)	NS
Leukocytes: femur (x10^5/mg)	2.2 ± 0.78 (n=6)	2.76 ± 0.60 (n=5)	NS
Spleen (mg)	90.2 ± 19.24 (n=15)	87.4 ± 25.12 (n=17)	NS
Spleen: body weight (mg/g)	3.7 ± 0.62 (n=17)	4.4 ± 1.01 (n=17)	<0.05
Leukocytes: spleen (x10^5/mg)	10.4 ± 3.11 (n=12)	9.0 ± 3.37 (n=14)	NS

Table 1; Murine body and organ characteristics. Table of measurements from naïve male and female mice. Data are displayed as mean ± SEM of n number of experiments, as displayed in parentheses. Female data were compared to male by Student's *t*-test and *p* value displayed. NS, not significant.

Figure 1

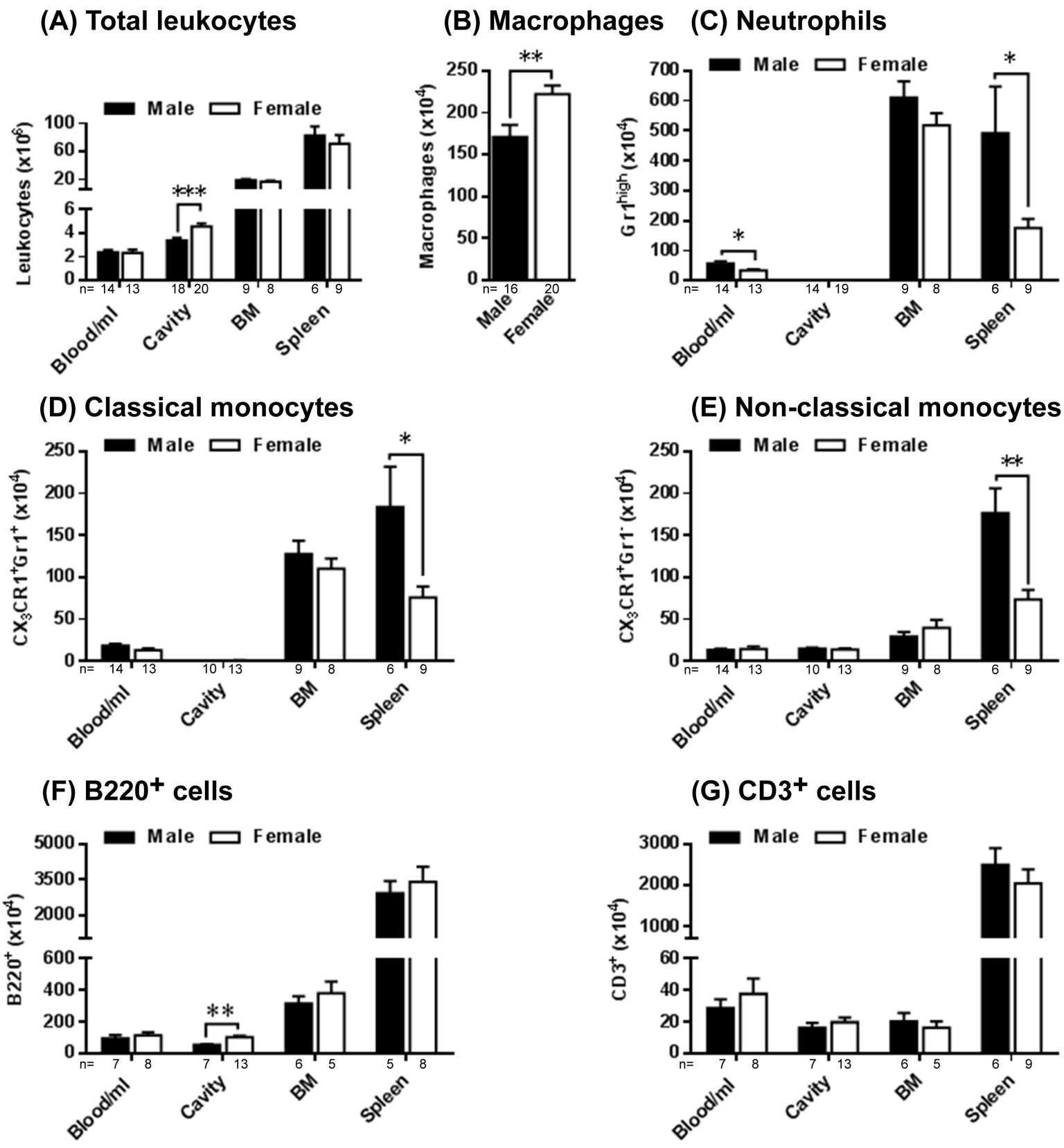

(A) Total leukocytes
Male
Female
Leukocytes (x10^6)
100
60
20
6
4
2
0
***
n= 14 13 18 20 9 8 6 9
Blood/ml
Cavity
BM
Spleen
(B) Macrophages
Macrophages (x10^4)
250
200
150
100
50
0
**
n= 16 20
Male
Female
(C) Neutrophils
Male
Female
Gr1^high (x10^4)
700
600
500
400
300
200
100
0
*
*
n= 14 13 14 19 9 8 6 9
Blood/ml
Cavity
BM
Spleen
(D) Classical monocytes
Male
Female
CX3CR1+Gr1+ (x10^4)
250
200
150
100
50
0
*
n= 14 13 10 13 9 8 6 9
Blood/ml
Cavity
BM
Spleen
(E) Non-classical monocytes
Male
Female
CX3CR1+Gr1- (x10^4)
250
200
150
100
50
0
**
n= 14 13 10 13 9 8 6 9
Blood/ml
Cavity
BM
Spleen
(F) B220+ cells
Male
Female
B220+ (x10^4)
5000
3500
2000
600
400
200
0
**
n= 7 8 7 13 6 5 5 8
Blood/ml
Cavity
BM
Spleen
(G) CD3+ cells
Male
Female
CD3+ (x10^4)
3000
2000
1000
60
40
20
0
n= 7 8 7 13 6 5 6 9
Blood/ml
Cavity
BM
Spleen

Figure 2

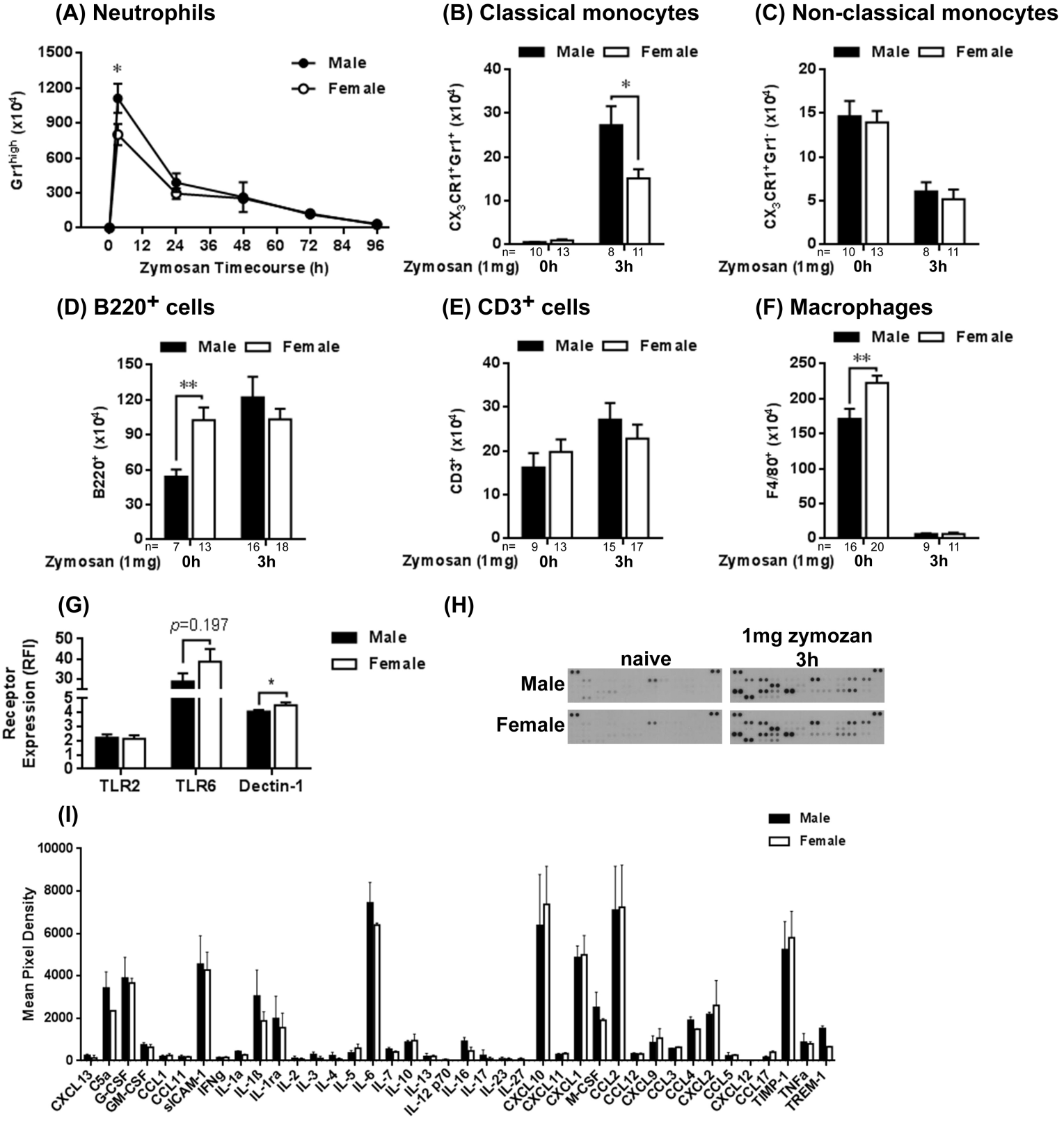

(A) Neutrophils
Male
Female
Gr1high (x10⁴)
Zymosan Timecourse (h)
(B) Classical monocytes
CX3CR1+Gr1+ (x10⁴)
Zymosan (1mg)
0h
3h
(C) Non-classical monocytes
CX3CR1+Gr1- (x10⁴)
(D) B220+ cells
B220+ (x10⁴)
(E) CD3+ cells
CD3+ (x10⁴)
(F) Macrophages
F4/80+ (x10⁴)
(G)
p=0.197
Receptor Expression (RFI)
TLR2
TLR6
Dectin-1
(H)
naive
1mg zymozan 3h
(I)
Mean Pixel Density
CXCL13
C5a
G-CSF
GM-CSF
CCL1
CCL11
sICAM-1
IFNg
IL-1a
IL-1ß
IL-1ra
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-10
IL-13
IL-12 p70
IL-16
IL-17
IL-23
IL-27
CXCL10
CXCL11
CXCL1
M-CSF
CCL2
CCL12
CXCL9
CCL3
CCL4
CXCL2
CCL5
CXCL12
CCL17
TIMP-1
TNFa
TREM-1

Figure 3

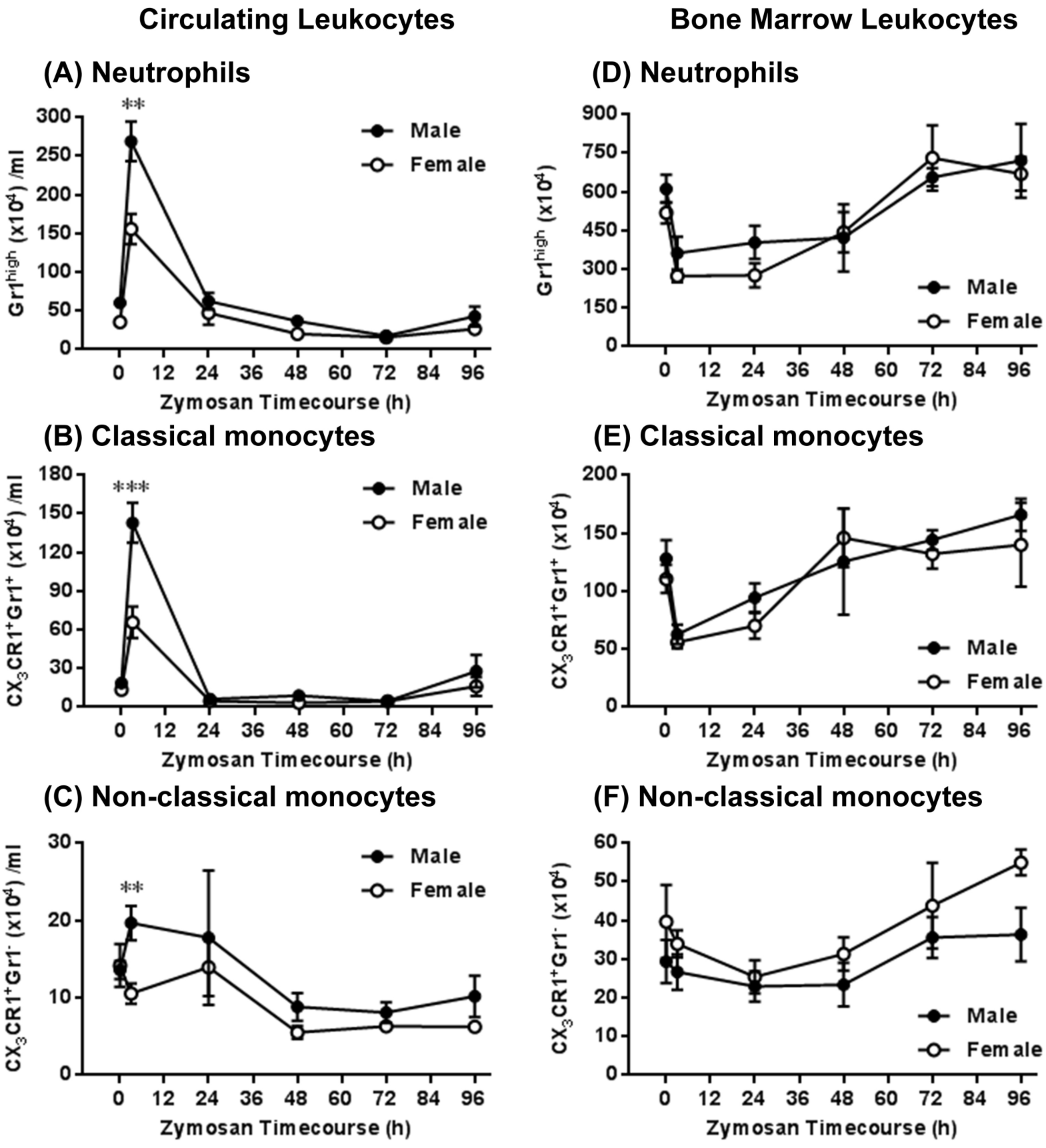

Circulating Leukocytes
Bone Marrow Leukocytes
(A) Neutrophils
(D) Neutrophils
(B) Classical monocytes
(E) Classical monocytes
(C) Non-classical monocytes
(F) Non-classical monocytes
Male
Female
Zymosan Timecourse (h)
Gr1high (x10⁴) /ml
Gr1high (x10⁴)
CX3CR1+Gr1+ (x10⁴) /ml
CX3CR1+Gr1+ (x10⁴)
CX3CR1+Gr1- (x10⁴) /ml
CX3CR1+Gr1- (x10⁴)
**
***
**

Figure 4

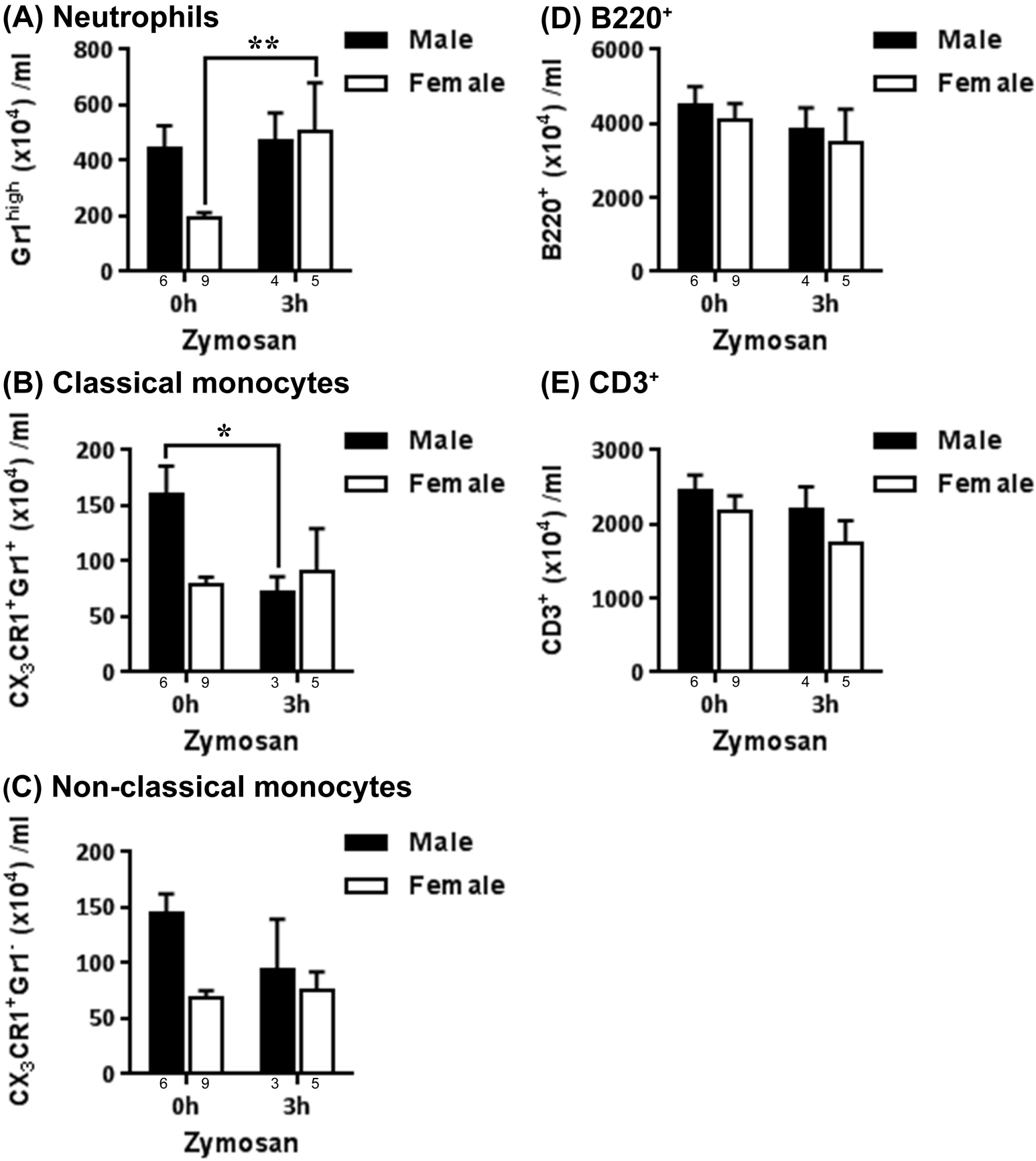
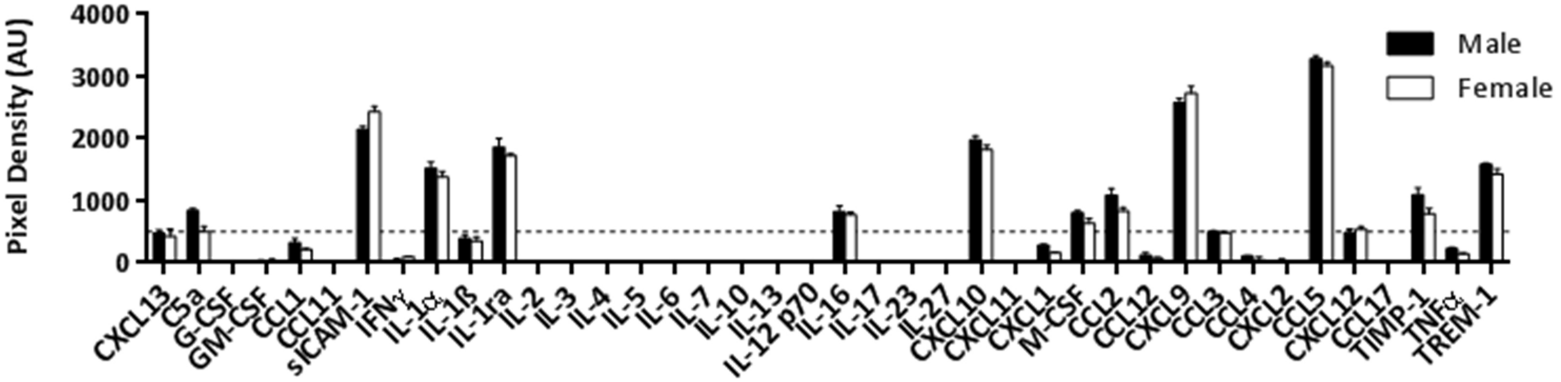
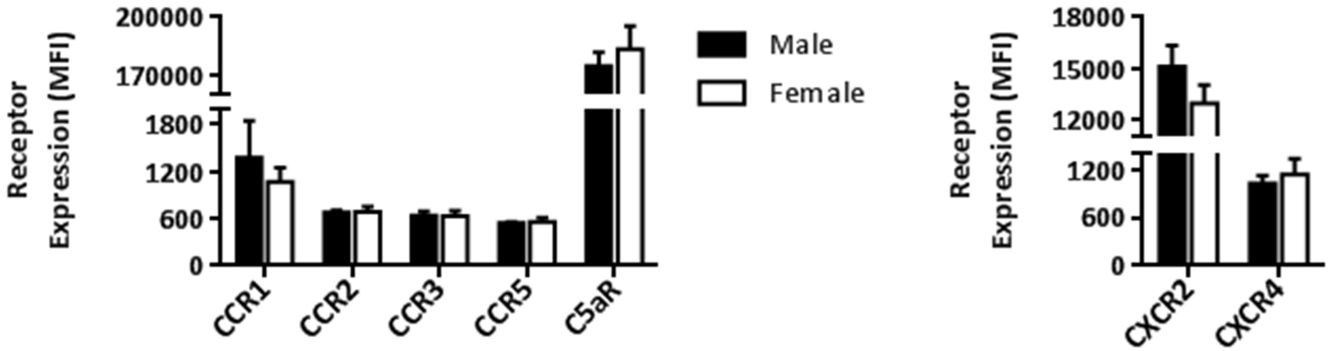

(A) Neutrophils
Gr1high (x10^4) /ml
800
600
400
200
0
**
Male
Female
6
9
4
5
0h
3h
Zymosan
(D) B220+
B220+ (x10^4) /ml
6000
4000
2000
0
Male
Female
6
9
4
5
0h
3h
Zymosan
(B) Classical monocytes
CX3CR1+Gr1+ (x10^4) /ml
200
150
100
50
0
*
Male
Female
6
9
3
5
0h
3h
Zymosan
(E) CD3+
CD3+ (x10^4) /ml
3000
2000
1000
0
Male
Female
6
9
4
5
0h
3h
Zymosan
(C) Non-classical monocytes
CX3CR1+Gr1- (x10^4) /ml
200
150
100
50
0
Male
Female
6
9
3
5
0h
3h
Zymosan

Figure 5

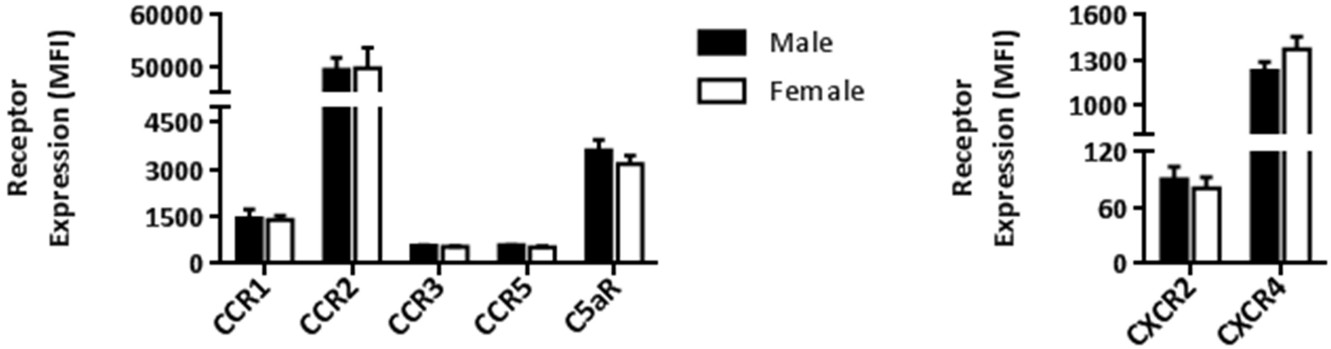
(A) Naive spleen cytokine micro-environment



(B) Splenic neutrophils



(C) Splenic classical monocytes



(D) Splenic non-classical monocytes

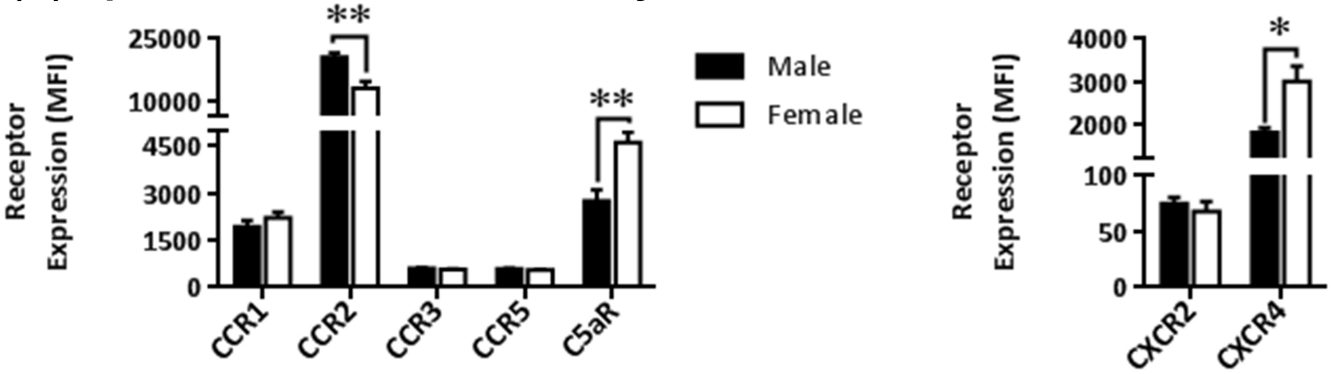
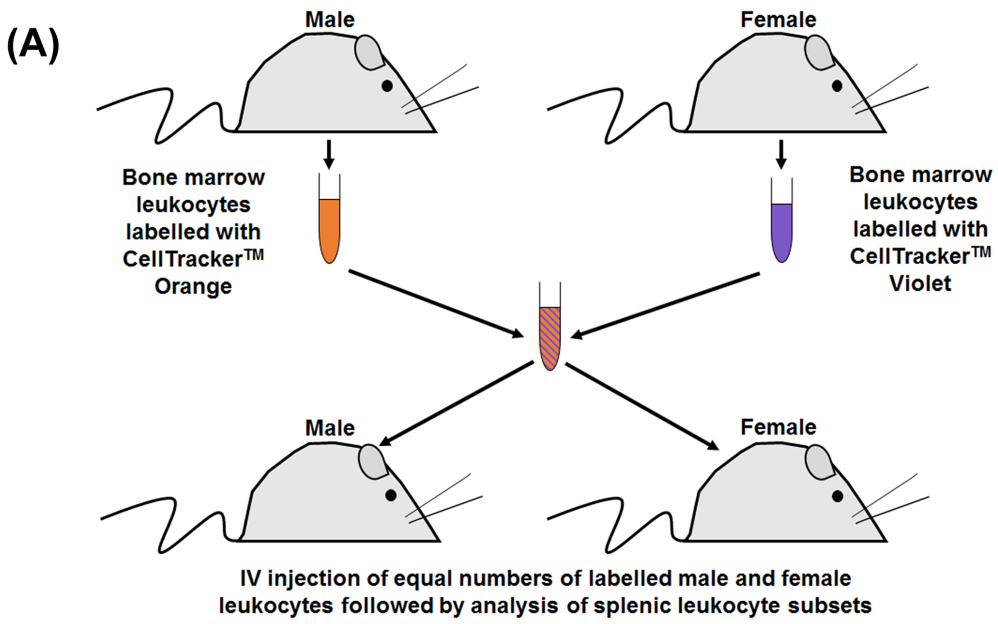


Figure 6


(A)
Male
Female
Bone marrow leukocytes labelled with CellTracker™ Orange
Bone marrow leukocytes labelled with CellTracker™ Violet
Male
Female
IV injection of equal numbers of labelled male and female leukocytes followed by analysis of splenic leukocyte subsets

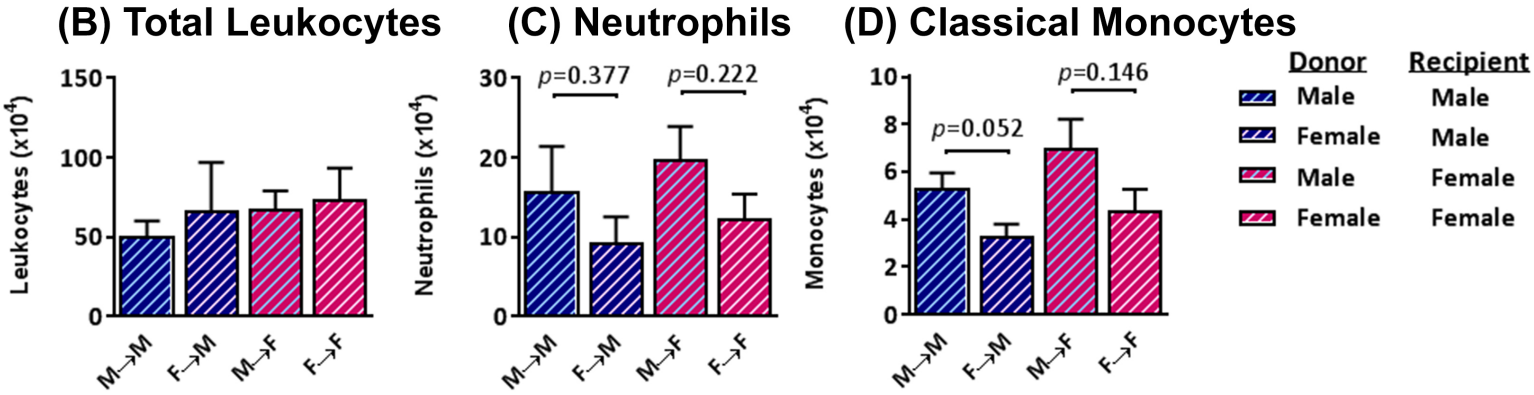
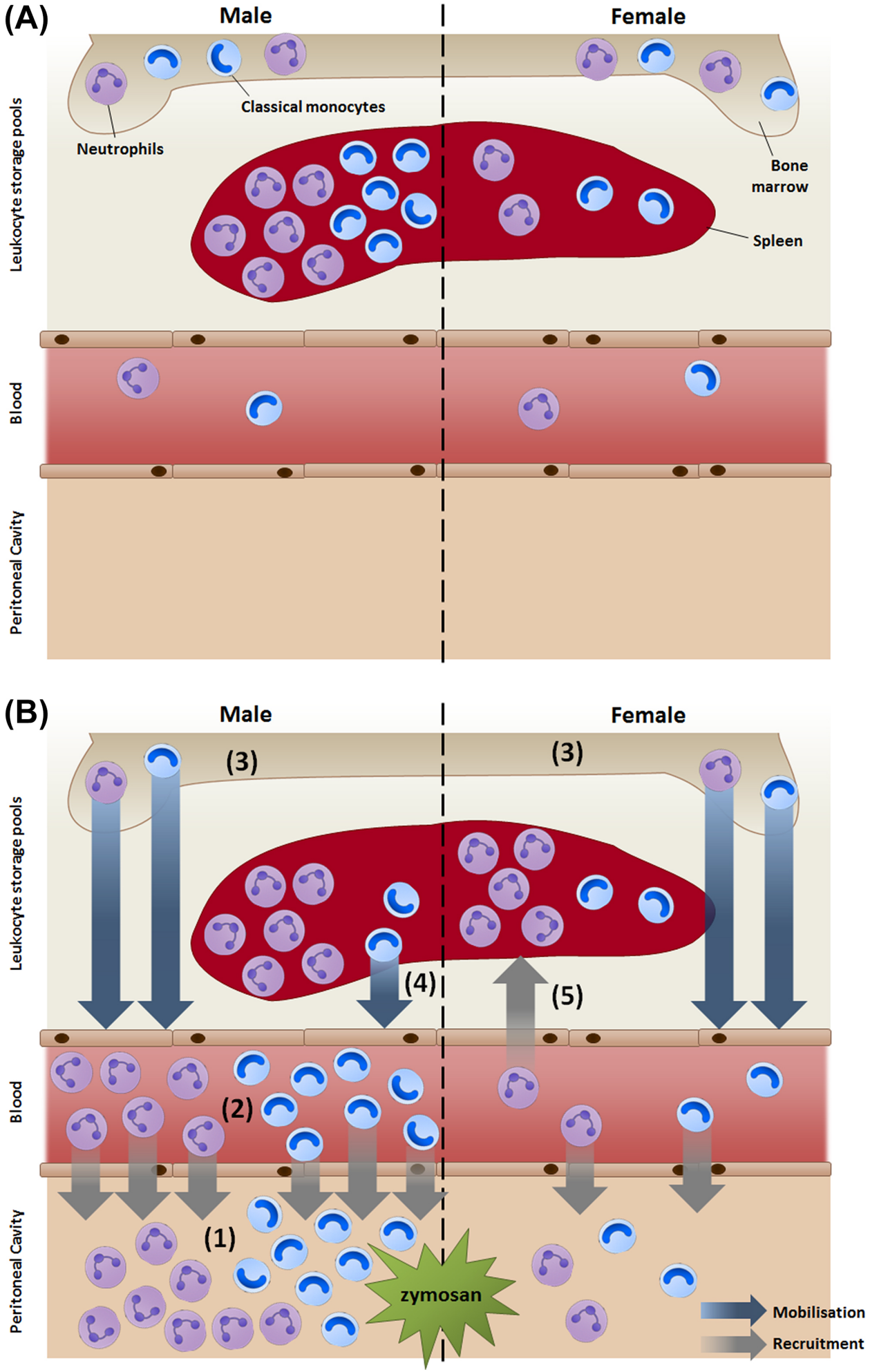

(B) Total Leukocytes
Leukocytes (x10⁴)
150
100
50
0
M→M
F→M
M→F
F→F
(C) Neutrophils
Neutrophils (x10⁴)
30
20
10
0
p=0.377
p=0.222
(D) Classical Monocytes
Monocytes (x10⁴)
10
8
6
4
2
0
p=0.052
p=0.146
Donor
Recipient
Male
Male
Female
Male
Male
Female
Female
Female

Figure 7


(A)
Male
Female
Classical monocytes
Neutrophils
Bone marrow
Spleen
Leukocyte storage pools
Blood
Peritoneal Cavity
(B)
Male
Female
(3)
(3)
(4)
(5)
(2)
(1)
zymosan
Leukocyte storage pools
Blood
Peritoneal Cavity
Mobilisation
Recruitment